

Serum 25-hydroxyvitamin D, metabolome, and bronchiolitis severity among infants—A multicenter cohort study

To the Editor:

Bronchiolitis is the most common lower respiratory infection in infants and the leading cause of infant hospitalizations in the United States (approximately 130 000 hospitalizations annually).¹ Prior research has linked vitamin D status to bronchiolitis pathobiology. Indeed, 25-hydroxyvitamin D (25OHD) undergoes enzymatic conversions to its biologically active form, 1,25-dihydroxyvitamin D (1,25OH₂D), in immune cells (eg, macrophages, dendritic cells, and T cells), and plays a pleiotropic regulatory role on both innate and adaptive immune systems.²

Epidemiological investigations have reported the associations of lower blood 25OHD levels at birth or early infancy with an increased risk and severity of acute respiratory infections (ARIs), including bronchiolitis.² Similarly, a 2017 meta-analysis of individual participant data from 25 trials demonstrated that vitamin D supplementation is protective against ARIs in subjects with low baseline 25OHD concentrations.³ While these earlier reports have evaluated circulating 25OHD levels in relation to disease processes,^{2,3} it remains unclear how vitamin D interacts with the numerous downstream functional molecules that can influence clinical outcomes. Metabolomics, a high-throughput technology, addresses this knowledge gap through comprehensively characterizing small-molecule metabolites that are a function of the infant's genetic makeup and environmental factors,⁴ such as vitamin D status.

In this study, by testing the serum metabolome from a nested subset of a multicenter bronchiolitis cohort study, we investigated the relationships between serum 25OHD levels, metabolome, and disease severity. More specifically, we tested the hypothesis that 25OHD-related serum metabolites are associated with the risk of positive pressure ventilation (PPV) use among infants with bronchiolitis.

This is an analysis of the data from a multicenter prospective cohort study of infants with severe bronchiolitis (ie, bronchiolitis requiring hospitalization). Details of the study design, setting, subjects, data measurement, and analysis may be found in the Data S1. In brief, the 35th Multicenter Airway Research Collaboration (MARC-35) cohort study⁵ enrolled 1016 infants (age <1 year) hospitalized with bronchiolitis at 17 sites across 14 states of United States (Table S1) during the 2011–2014 winter seasons. Bronchiolitis was defined using the American Academy of Pediatrics guidelines—acute respiratory illness with some combination of rhinitis, cough, tachypnea, wheezing, crackles, and retractions.⁶ We excluded patients who were transferred to a participating hospital >24 hours after the original hospital admission, those who were consented >24 hours

after admission, or those with known heart-lung disease, immunodeficiency, immunosuppression, or gestational age <32 weeks. The institutional review board of each participating hospital approved the study. We obtained written informed consent from the infant's parent or guardian.

In addition to the collection of phenotypic data via structured interview and chart review, blood specimens were collected within 24 hours of hospitalization using a standardized protocol.⁵ Serum total 25OHD levels were quantified by immunoassays; bioavailable and free 25OHD levels were computed using previously validated formulas. Serum metabolites (metabolome) were profiled using ultra-high performance liquid chromatography-tandem mass spectrometry by Metabolon (Durham, NC, USA). The details of the tests and quality control measures may be found in Data S1. The clinical outcome of interest was the use of PPV—defined as use of continuous positive airway pressure and/or intubation during hospitalization.⁵

For the present analysis, we selected 140 infants who had sufficient volume of serum specimens and performed serum metabolomic profiling. First, to derive a subset of serum metabolites associated with the circulating 25OHD (3 components—total, bioavailable, and free) levels, we constructed sparse partial least squares (sPLS) regression models with cross-validation and LASSO regularization that minimize potential overfitting. The sPLS method has advances that it handles correlated variables (eg, metabolites) and that it can also simultaneously model multiple response variables (total, bioavailable, and free 25OHD levels in this study). To determine the association between each of these selected serum metabolites and the risk of PPV use, we next constructed multivariable logistic regression and random-effects models adjusting for age, sex, history of premature birth, feeding status, weight at presentation, in-hospital corticosteroid use, detected virus, and potential patient clustering within the hospitals. *P*-values were adjusted for multiple testing with the Benjamini-Hochberg false discovery rate (FDR) method. All analyses were conducted in R version 3.4.

The analytic (*n* = 140) and non-analytic (*n* = 876) cohorts were not significantly different for most patient characteristics, including the serum 25OHD levels (*P* > .05; Table S2), except the analytic cohort had a lower proportion of respiratory syncytial virus infection and higher proportion of rhinovirus infection (both *P* < .05). Of 140 infants in the analytic cohort, the median age was 3 months (interquartile range [IQR] 1–6 months), 62% were male, and 39% were non-Hispanic white. The median level of serum total 25OHD was 24.8 ng/mL (IQR 15.9–32.8 ng/mL). Its level was significantly correlated with that of bioavailable 25OHD (*r* = .73; *P* < .001) and free

TABLE 1 Patient characteristics of infants hospitalized for bronchiolitis according to serum total 25-hydroxyvitamin D level

Variables	Total 25OHD <24.8 ng/mL (n = 70)	Total 25OHD ≥24.8 ng/mL (n = 70)	P-value
Characteristics			
Age (mo), median, (IQR)	1.9 (0.9-3.5)	5.0 (2.6-8.0)	<.001
Male sex	45 (64.3)	42 (60.0)	.73
Race/ethnicity			
Non-Hispanic white	25 (35.7)	29 (41.4)	.56
Non-Hispanic black	13 (18.6)	16 (22.9)	
Hispanic	29 (41.4)	24 (34.3)	
Other	3 (4.3)	1 (1.4)	
Parental history of asthma	23 (32.9)	21 (30.4)	.90
Maternal smoking during pregnancy	8 (11.4)	9 (13.0)	.98
C-section delivery	23 (32.9)	29 (42.0)	.35
Prematurity (32-37 wk)	17 (24.3)	17 (24.3)	.99
Previous breathing problems before the index hospitalization ^a	11 (15.7)	23 (32.9)	.03
History of eczema	8 (11.4)	12 (17.1)	.47
Mostly breastfed for the first 3 mo of age	40 (59.7)	26 (41.3)	.054
Smoke exposure at home	4 (5.7)	11 (15.7)	.10
Clinical presentation			
Weight at presentation (kg), median (IQR)	5.1 (4.0-6.5)	7.2 (5.5-8.4)	<.001
Respiratory rate at presentation (per min), median (IQR)	46 (40-60)	52 (44-62)	.03
Oxygen saturation at presentation			
<90%	12 (16.2)	7 (8.7)	.32
90%-93%	10 (14.7)	16 (23.2)	
≥94%	47 (68.1)	47 (68.1)	
Received corticosteroids during pre-hospitalization visit	8 (11.4)	8 (11.4)	.99
Virology			
Sole RSV infection	35 (50.0)	28 (40.0)	.31
Sole rhinovirus infection	12 (17.1)	14 (20.0)	.83
RSV + rhinovirus coinfection	5 (7.1)	7 (10.0)	.76
Others	18 (25.7)	21 (30.0)	.57
Laboratory data			
Serum bioavailable 25OHD (ng/mL), median (IQR)	2.6 (1.7-3.7)	5.2 (4.0-8.1)	<.001
Serum free 25OHD (pg/mL), median (IQR)	8.4 (5.4-12.2)	15.5 (10.9-23.8)	<.001
Serum vitamin D-binding protein (μg/mL), median (IQR)	134.6 (85.1-170.6)	161.3 (103.5-223.1)	.03
Serum LL-37 (ng/mL), median (IQR)	36.0 (28.0-55.5)	48.0 (28.5-65.5)	.23
Food sensitization ^b	10 (14.3)	16 (22.9)	.28
Aeroallergen sensitization ^b	0 (0)	2 (2.9)	.48
Hospital course			
Positive pressure ventilation ^c	25 (35.7)	13 (18.6)	.04
Admission to intensive care unit	39 (55.7)	31 (44.3)	.24
Hospital length-of-stay ≥3 d	39 (55.7)	33 (47.1)	.40
Hospital length-of-stay (d), median (IQR)	3 (2-8)	2 (2-5)	.04

IQR, interquartile range; RSV, respiratory syncytial virus; 25OHD, 25-hydroxyvitamin D.

Data are no. (%) of infants unless otherwise indicated.

^aDefined as a child having cough that wakes him or her at night and/or causes emesis, or when the child has wheezing or shortness of breath without cough.^bDefined by having 1 or more positive values for allergen-specific IgE.^cDefined as continuous positive airway pressure and/or intubation during inpatient stay, regardless of location, during the index hospitalization.

25OHD ($r = .66$; $P < .001$; Figure S1). Most patient characteristics did not differ significantly by total 25OHD status (Table 1), while infants with lower total 25OH levels were younger, less likely to have a previous breathing problem, and had a lower weight at presentation (all $P < .05$). Additionally, these infants had a higher likelihood of PPV use and longer hospital length-of-stay (both $P < .05$) than those with higher total 25OHD levels.

Metabolomics testing of serum specimens detected a total of 707 named metabolites from 96 subpathways in 8 superpathways. Based on the sPLS ordination, the overall metabolome profiles clustered by total 25OHD status (Figures 1 and S2). By computing their loading scores with the sPLS model, we identified a set of top 20 serum metabolites that were most strongly associated with the serum 25OHD (total, bioavailable, and free) levels (Figures 2 and S3). These metabolites were products of predominantly lipid metabolism (11 metabolites), followed by amino acid/peptide, nucleotide, and carbohydrate metabolism. In the multivariable logistic regression models adjusting for potential confounders and the random-effects models accounting for hospital effects, 9 of these 20 metabolites were significantly associated with the risk of PPV use (all FDR < 0.05). Specifically, among the metabolites that were correlated with lower 25OHD levels, 3 metabolites—fibrinopeptide A, N1-methyladenosine, and sphingomyelin (d18:1/18:1, d18:2/18:0)—were associated with a significantly higher risk of PPV use (all FDR < 0.05 ; Figure 2). By contrast, among the metabolites that were correlated with higher 25OHD levels, 6 metabolites—1-(1-enyl-stearoyl)-2-oleoyl-glycerophosphorylethanolamine (GPE), 1-(1-enyl-stearoyl)-2-linoleoyl-GPE, isoursodeoxycholate, 1-linoleoyl-glycerophosphate (GPA), glycerate, and glycylvaline—were associated with a significantly lower risk of PPV use (all FDR < 0.05). The discrimination ability of the 20 selected metabolites on the risk of PPV use was high, with an area-under-the receiver operating characteristic curve of 0.92 (Figure S4).

Based on the data from a prospective multicenter cohort of infants hospitalized for bronchiolitis, serum 25OHD levels were associated with differences in the serum metabolome profile—a metabolomic signature. Specifically, from the 707 named metabolites detected, we identified a set of 20 metabolites (predominantly lipids) that were associated with circulating 25OHD (total, bioavailable, and free) levels. Among these, metabolites correlated with lower 25OHD (eg, sphingolipids) were associated with a significantly higher risk of PPV use. In contrast, metabolites correlated with higher 25OHD (eg, plasmalogen pathway metabolites [GPE and GPA]) were associated with a significantly lower risk of PPV use. This is the first investigation that has examined the interrelationships between circulating 25OHD, metabolome, and clinical outcomes among infants with bronchiolitis.

Consistent with our observations, earlier reports of several disease conditions, including wheezing in young children, have found that the circulating total 25OHD concentrations were associated with specific blood and urine metabolomic profiles, particularly phospholipids.^{7,8} For instance, a recent nested case-control study of 84 children with recurrent wheeze (or asthma) and 161 controls

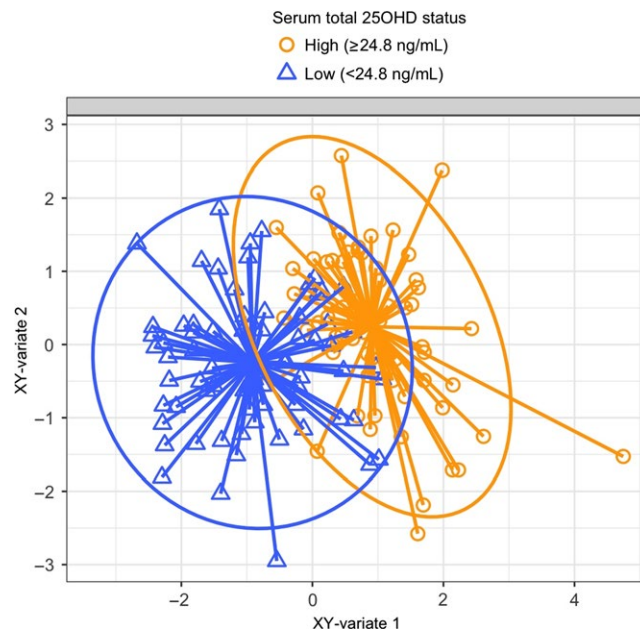


FIGURE 1 Sparse partial least squares analysis score plot of serum metabolome according to total 25-hydroxyvitamin D levels. Each dot represents the global serum metabolomic profile of a single infant, by plotting the component scores in the smaller subspace spanned by latent variables of sparse partial least squares (sPLS) model. The scatter plot indicates the similarities and dissimilarities in the global metabolome profile between infants, grouped by serum total 25OHD status (defined by the median values). The ellipses are 95% confidence intervals. The arrows start from the centroid of each group and end for each patient belonging to each group

reported that, at age 3 years, the sphingolipid and glycerophospholipid (including GPE) metabolism pathways are associated with both serum 25OHD levels and clinical outcomes.⁷ The literature has also demonstrated that 1,25OH₂D influences the metabolism of sphingolipids by regulating multiple components (eg, the expression of sphingosine 1-phosphate [S1P] phosphatase, S1P receptors, and sphingomyelinase).⁹ Sphingolipids are not only building blocks of the cell membrane bilayer but also serve as signaling molecules with important roles in immune response and the pathobiology of inflammatory diseases (eg, asthma and autoimmune diseases).⁹ Emerging evidence has also shown that an elevated level of sphingolipids in the upper airway is associated with higher severity in infants with bronchiolitis.⁵

This study also demonstrated that glycerophospholipids, particularly plasmalogen pathway metabolites (eg, GPE and GPA), were associated with both higher circulating 25OHD levels and lower risk of PPV use. Consistent with this finding, a cohort study of 392 Finnish adults demonstrated the relationships between serum 25OHD levels and these lipids in the plasmalogen pathway.⁸ Plasmalogen is not only a major structural component of cell membrane and pulmonary surfactant, but also plays biologic roles (eg, generation of inflammatory mediators and protection against oxidation of surfactant lipids).¹⁰ The literature has also demonstrated

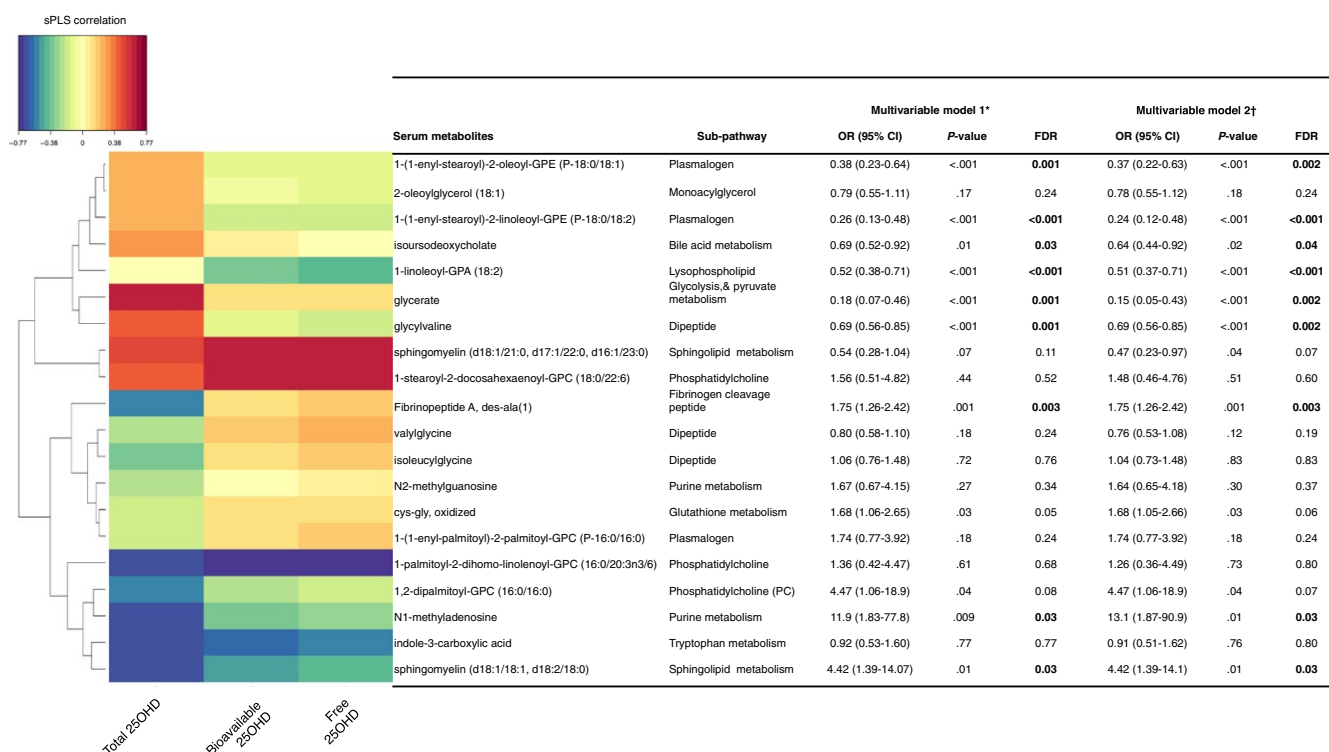


FIGURE 2 Correlations between serum 25-hydroxyvitamin D (25OHD) and selected serum metabolites, and their associations with the risk of positive pressure ventilation use among infants hospitalized with bronchiolitis. The heatmap (left) visualizes the correlations between the serum 25OHD (total, bioavailable, and free) levels and 20 selected metabolites that were examined using hierarchical clustering with an average linkage algorithm. The table (right) shows the adjusted associations between each of selected serum metabolites with the risk of PPV use. Bold results are statistically significant with FDR of <0.05. OR is calculated per each incremental increase in scaled level. 25OHD, 25-hydroxyvitamin D; CI, confidence interval; FDR, false discovery rate; GPA, glycerophosphate; GPC, glycerophosphocholine; GPE, glycerophosphorylethanolamine; OR, odds ratio; sPLS, partial least squares. *Logistic regression models adjusting for patients' age, sex, history of premature birth, feeding status, weight at presentation, corticosteroid use, and detected virus. †Random-effects models adjusting for the covariates above and accounting for potential patient clustering within the hospitals [Colour figure can be viewed at wileyonlinelibrary.com]

that levels of plasmalogen are insufficient in premature infants with bronchopulmonary dysplasia and smokers with COPD.¹⁰ Our study corroborates these earlier reports and extends them by demonstrating the interrelations between circulating 25OHD, metabolome, and disease severity in infants with bronchiolitis.

We acknowledge several potential limitations to the study. First, 25OHD and metabolites were measured early in the hospital course of critical illness at a single time point and from a single bio-fluid (ie, serum). Second, free and bioavailable 25OHD levels were not directly measured but computed using previously validated formulas. Additionally, between-assay variability of total 25OHD measurement is possible because of the lack of standardization. Third, this study did not have comparative metabolic information from a "healthy control" group of 25OHD-sufficient infants. Nonetheless, the study aim was not to evaluate the associations between 25OHD, metabolome, and the development of bronchiolitis, but to examine their contributions to acute severity among infants hospitalized for bronchiolitis. Fourth, we were unable to fully account for reverse causation and potential confounding, such as differences in intensive care resources. Yet, we statistically accounted for potential clustering of patients at the hospital level. Fifth, while we performed cross-validations and LASSO regularization to limit potential

overfitting, validation in a separate population is required to confirm our inferences. The findings are, however, consistent with recent publications on related topics.^{7,8} Finally, while the study population consisted of a racially/ethnically and geographically diverse sample, all were hospitalized for bronchiolitis and, as such, the findings may not be generalizable to children with mild-to-moderate bronchiolitis. Still, our inference remains directly relevant to 130 000 hospitalized US children each year.¹

In sum, based on the multicenter data of 140 infants hospitalized for bronchiolitis, we found that serum 25OHD levels were associated with a set of serum metabolites—a metabolomic signature. Among these, the metabolites correlated with lower 25OHD levels (eg, sphingomyelin) were associated with a significantly higher risk of PPV use, while those correlated with higher 25OHD levels (eg, plasmalogen pathway metabolites) were associated with a lower risk. Although causality remains to be elucidated, prior research and our observations collectively suggest that circulating 25OHD, through affecting metabolites associated with inflammatory processes, may influence bronchiolitis severity. Our findings advance research into the complex interrelations between the environmental factors (eg, vitamin D), host immunity, and the pathobiology of bronchiolitis in infants.

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CONFLICT OF INTEREST

The authors have no financial relationships relevant to this article to disclose.

AUTHOR CONTRIBUTIONS

KH, CJS, and CAC conceived the study. JCC, JMM, and CT contributed to data collection and management. CAC obtained research funding and supervised the conduct of the study. CJS contributed to the metabolomics processing and testing. KH analyzed the data and drafted the manuscript. All authors contributed substantially to its revision. KH takes responsibility for the paper as a whole.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

ONLINE SUPPLEMENT

Serum 25-hydroxyvitamin D, metabolome, and bronchiolitis severity among infants:

A multicenter cohort study

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Supplemental Methods

Table S1. Principal Investigators at the 17 Participating Sites in MARC-35

Table S2. Characteristics of Analytic and Non-analytic Cohorts in MARC-35

Figure S1. Correlations of Serum Total 25-hydroxyvitamin D (25OHD) with Bioavailable and Free 25OHD Levels

Figure S2. Sparse Partial Least Squares Analysis Score Plots of Serum Metabolome According to 25-hydroxyvitamin D Levels

Figure S3. Loading Plot of Serum Metabolites Related to Lower Serum 25-hydroxyvitamin D Levels Among Infants with Bronchiolitis

Figure S4. Receiver Operating Characteristic Curve of the 20-metabolite Model on the Risk of Positive Pressure Ventilation Use

Data S1: SUPPLEMENTAL METHODS

Study Design, Setting, and Participants

This is an analysis of data from a multicenter prospective cohort study of infants hospitalized with bronchiolitis (severe bronchiolitis). This cohort study, called the 35th Multicenter Airway Research Collaboration (MARC-35),^{1,2} was coordinated by the Emergency Medicine Network (EMNet), a collaboration of 245 participating hospitals.³

Using a standardized protocol, investigators at 17 sites across 14 U.S. states (**Table E1**) enrolled infants (aged <12 months) hospitalized with an attending physician diagnosis of bronchiolitis during three consecutive bronchiolitis seasons (from November 1 through April 30) during 2011-2014. Bronchiolitis was defined by the American Academy of Pediatrics guidelines – acute respiratory illness with some combination of rhinitis, cough, tachypnea, wheezing, crackles, and retractions.⁴ We excluded infants who were transferred to a participating hospital >24 hours after the original hospitalization, those who were consented >24 hours after hospitalization, or those with known heart-lung disease, immunodeficiency, immunosuppression, or gestational age <32 weeks. All patients were treated at the discretion of the treating physician. The institutional review board at each of the participating hospitals approved the study. Written informed consent was obtained from the parent or guardian.

Data Collection

Investigators conducted a structured interview that measured patients' socio-demographic characteristics, medical, family, and environmental history, and details of the acute illness. Emergency department and hospital medical record reviews provided further clinical data, such as vital signs, physical examination, medical management, and disposition. All data were

reviewed at the EMNet Coordinating Center (Boston, MA, USA), and site investigators were queried about missing data and discrepancies identified by manual data checks. In addition to the clinical data, site investigators collected blood samples within 24 hours of hospitalization and stored the samples at -80°C using a standardized protocol.^{1,2} For the present analysis, we selected 140 infants who had sufficient volume of serum specimens, with overrepresentation of rhinovirus infection (**Table E2**). Their serum samples were tested for: 1) vitamin D-related compounds using immunoassays, and 2) metabolites (metabolome) using ultra-high performance liquid chromatography-tandem mass spectrometry (UPLC-MS).

Serum Total 25-hydroxyvitamin D and Vitamin D-binding Protein Measurement

Serum total 25-hydroxyvitamin D (25OHD) concentration was quantified by using the Abbott ARCHITECT 25-OH Vitamin D chemiluminescent microparticle immunoassay (Abbott Park, IL, USA). The lower limit of detection of the 25OHD assay is 1.9 ng/ml, with an intra-assay and inter-assay coefficient of variations of $<4.0\%$. In addition, serum vitamin D-binding protein (DBP) concentration was quantified using a quantitative sandwich enzyme-linked immunosorbent assay (Quantikine Human Vitamin D Binding Protein Immunoassay, Minneapolis, MN, USA). The lower limit of detection of the DBP assay is 0.7 ng/ml, with an intra-assay and inter-assay coefficient of variations of $<7.5\%$.

Because 25OHD in serum is bound to DBP (85%-90%) and albumin (10%-15%), $<1\%$ of serum 25OHD exists in its free form.⁵ Only 25OHD that is liberated from these binding proteins enter cells and regulate intracellular biologic function. Based on previously-validated formulas in the literature,⁵⁻⁷ free 25OHD levels were computed by using the serum concentrations of total 25OHD, DBP, and albumin, as well as binding-affinity constants for 25OHD's interaction with

these binding proteins. In addition, as 25OHD bound to albumin is deemed to have biological activity, the sum of free- and albumin-bound 25OHD was referred to as bioavailable 25OHD.^{6,7} The correlations of serum concentrations of total 25OHD with computed free 25OHD and bioavailable 25OHD are shown in **Figure E1**.

Serum Metabolomic Profiling and Quality Control

Serum metabolomic profiling used 100 µl of serum sample. All samples were blinded to Metabolon (Durham, NC, USA) and processed in a random order. The metabolic profiling used ACQUITY UPLC (Waters, Milford, MA, USA) and Q-Exactive high resolution/accurate MS interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution (Thermo Fisher Scientific, Waltham, MA, USA).

Sample preparation was carried out as described previously.¹¹ In brief, recovery standards were added prior to the first step in the extraction process for quality control purposes. Proteins were precipitated with methanol under vigorous shaking for 2 minutes (Glen Mills Genogrinder 2000; Clifton, NJ, USA) followed by centrifugation. The resulting extract was divided into four fractions. The first aliquot was analyzed using acidic positive ion conditions, chromatographically optimized for more hydrophilic compounds. The second aliquot was also analyzed using acidic positive ion conditions, but was chromatographically optimized for more hydrophobic compounds. The third aliquot was analyzed with basic negative ion optimized conditions using a separate dedicated C18 column. The fourth aliquot was analyzed via negative ionization following elution from a HILIC column (Waters UPLC BEH Amide). The MS analysis alternated between MS and data-dependent MSⁿ scans using dynamic exclusion.

Metabolites were identified by automated comparison of the ion features in the experimental samples to a reference library of chemical standard entries that include retention time, molecular weight (m/z), preferred adducts, and in-source fragments as well as associated MS spectra, and curated by visual inspection for quality control using QUICS software.¹² Identification of known chemical entities was based on comparisons to metabolomic library entries of >3,000 purified standards. Peaks were quantified using area-under-the-curve. Raw area counts for each metabolite in each sample were normalized to correct for variation due to instrument inter-day tuning differences by the median value for each run-day, setting the medians to 1.0 for each run. Missing values were imputed with the observed minimum for that particular compound.

Four types of quality controls were analyzed in concert with the specimens: 1) samples generated from a pool from a small portion of each experimental specimen that served as technical replicate; 2) extracted water samples that served as process blanks; 3) samples of solvent used in extraction; and 4) a cocktail of standards spiked into every analyzed specimen that allowed instrument performance monitoring. The median relative standard deviation (RSD) for the standards that are added to each sample – a measure of instrument variability – was <5%.

IgE Measurement

Serum specific IgE (sIgE) was measured using two different assays (ImmunoCAP sIgE and ImmunoCAP ISAC) at the Phadia Immunology Reference Laboratory (Portage, MI, USA). The sIgE allergen assays conducted were milk, egg white, peanut, cashew nut, and walnut; a positive test was defined as ≥ 0.35 kU/L. The ImmunoCAP ISAC microarray immunoassay measures IgE antibodies to 112 components from 51 allergen sources including foods (e.g., eggs,

cow's milk, fish, shellfish, tree nuts, peanuts, soybeans, wheat, and some fruits) and aeroallergens (e.g., grasses, weeds, trees, animals [cat, dog, horse, and mouse], molds, dust mites, and cockroach). A positive result was defined as ≥ 0.30 ISU-E (ISAC Standardized Units); ISU-E provides an indication of IgE levels and is standardized to ImmunoCAP sIgE units.

Clinical Outcome Measure

The clinical outcome of interest was the use of positive pressure ventilation (PPV) – defined as the use of continuous positive airway pressure and/or intubation – at any time during the index hospitalization.^{2,13}

Statistical Analyses

We compared the patient characteristics, clinical presentation, virology, and laboratory data by dichotomized serum total 25OHD status – to simplify data presentation and interpretation – with the use of chi-square test or Wilcoxon-Mann-Whitney test as appropriate (**Table 1**). Total 25OHD levels were classified into two groups – low (< 24.8 ng/ml) and high (≥ 24.8 ng/ml) based on the median value.

Next, to examine the relationships of three serum vitamin D-related compounds (total, bioavailable, and free 25OHDs as the *continuous* variables) with serum metabolites, we fit a sparse partial least squares (sPLS) regression model by using *mixOmics* ver. 6.2.0¹⁴ implemented in the R statistical software (www.r-project.org). sPLS regression is a multivariate methodology that relates two continuous data matrices, such as serum 25OHD and metabolomic datasets. This method has advantages that it models multiple variables (e.g., total, bioavailable, and free 25OHDs), handles highly correlated variables (e.g., metabolomic data), and

simultaneously identifies latent variables.^{15,16} First, to examine the global serum metabolomic profile, we constructed cross-validated sPLS score plots that visualize between-individual similarities and dissimilarities in the global metabolomic profile. Next, to identify a subset of serum metabolites associated with circulating 25OHD (total, bioavailable, and free) levels, we also used sPLS regression (canonical mode) with LASSO regularization that minimizes potential overfitting. The model performance with respect to the sPLS dimensions and number of selected metabolites in the model was determined with 10-fold cross-validation repeated 100 times.

Lastly, to examine the associations of these selected serum metabolites with the risk of PPV, we constructed multivariable logistic regression models adjusting for potential confounders, such as patients' age, sex, history of premature birth (gestational age of <37 weeks), feeding status (mostly breastfeeding vs. formula feeding), body weight at presentation, corticosteroid use at the hospitalization, and detected virus. In addition, to account for potential patient clustering at the hospital level, we also constructed a random-effects model for each metabolite of interest. P-values were adjusted for multiple comparisons using the Benjamini-Hochberg false discovery rate (FDR) method.¹⁷ Analyses were conducted in R version 3.4.

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Table S1. Principal Investigators at the 17 Participating Sites in MARC-35

Amy D. Thompson, MD	Alfred I. duPont Hospital for Children, Wilmington, DE
Federico R. Laham, MD, MS	Arnold Palmer Hospital for Children, Orlando, FL
Jonathan M. Mansbach, MD, MPH	Boston Children's Hospital, Boston, MA
Vincent J. Wang, MD, MHA and Susan Wu, MD	Children's Hospital of Los Angeles, Los Angeles, CA
Michelle B. Dunn, MD and Jonathan M. Spergel, MD, PhD	Children's Hospital of Philadelphia, Philadelphia, PA
Juan C. Celedón, MD, DrPH	Children's Hospital of Pittsburgh, Pittsburgh, PA
Michael R. Gomez, MD, MS-HCA and Nancy Inhofe, MD	The Children's Hospital at St. Francis, Tulsa, OK
Brian M. Pate, MD and Henry T. Puls, MD	The Children's Mercy Hospital & Clinics, Kansas City, MO
Stephen J. Teach, MD, MPH	Children's National Medical Center, Washington, D.C.
Richard T. Strait, MD	Cincinnati Children's Hospital and Medical Center, Cincinnati, OH
Ilana Y. Waynik, MD	Connecticut Children's Medical Center, Hartford, CT
Sujit Iyer, MD	Dell Children's Medical Center of Central Texas, Austin, TX
Michelle D. Stevenson, MD, MS	Norton Children's Hospital, Louisville, KY
Wayne G. Shreffler, MD, PhD and Ari R. Cohen, MD	Massachusetts General Hospital, Boston, MA
Anne K Beasley, MD and Cindy S. Bauer, MD	Phoenix Children's Hospital, Phoenix, AZ
Thida Ong, MD	Seattle Children's Hospital, Seattle, WA
Charles G. Macias, MD, MPH	Texas Children's Hospital, Houston, TX

Table S2. Characteristics of Analytic and Non-analytic Cohorts in MARC-35

Variables	Analytic cohort n=140	Non-analytic cohort n=876	P-value
Characteristics			
Age (mo), median, (IQR)	3.1 (1.4-6.1)	3.2 (1.6-5.9)	0.74
Male sex	87 (62.1)	523 (59.7)	0.65
Race/ethnicity			0.21
Non-Hispanic white	54 (38.6)	376 (42.9)	
Non-Hispanic black	29 (20.7)	210 (24.0)	
Hispanic	53 (37.9)	255 (29.1)	
Other	4 (2.9)	35 (4.0)	
Parental history of asthma	44 (31.7)	301 (34.4)	0.58
Maternal smoking during pregnancy	17 (12.2)	130 (15.1)	0.62
C-section delivery	52 (37.4)	296 (34.4)	0.73
Prematurity (32-37 weeks)	34 (24.3)	152 (17.4)	0.06
Previous breathing problems before the index hospitalization*	34 (24.3)	172 (19.6)	0.25
History of eczema	20 (14.3)	129 (14.7)	0.99
Mostly breastfed for the first 3 months of age	66 (50.8)	357 (47.3)	0.52
Smoke exposure at home	15 (10.7)	141 (16.1)	0.13
Clinical presentation			
Weight at presentation (kg), median (IQR)	6.0 (4.4-7.8)	6.1 (4.8-7.7)	0.44
Respiratory rate at presentation (per minute), median (IQR)	48 (40-60)	48 (40-60)	0.58
Oxygen saturation at presentation			0.19
<90%	17 (12.4)	74 (8.5)	
90%-93%	26 (19.0)	129 (15.0)	
≥94%	94 (68.6)	655 (76.3)	
Received corticosteroids during pre-hospitalization visit	16 (11.4)	74 (8.5)	0.34
Virology			
Sole RSV infection	63 (45.0)	523 (59.7)	0.001
Sole rhinovirus infection	26 (18.6)	34 (3.9)	<0.001
RSV + rhinovirus coinfection	12 (8.6)	90 (10.3)	0.64
Others	39 (27.9)	229 (26.1)	0.67
Laboratory data			
Serum total 25OHD (ng/ml), median (IQR)	24.8 (15.9-32.8)	26.5 (18.9-33.1)	0.29
Serum bioavailable 25OHD (ng/ml), median (IQR)	3.8 (2.6-6.2)	4.3 (2.8-6.8)	0.12
Serum free 25OHD (pg/ml), median (IQR)	11.1 (8.2-19.5)	12.4 (8.3-19.0)	0.37
Serum vitamin D-binding protein (µg/ml), median (IQR)	42.3(93.6-195.4)	143.2 (94.1-199.5)	0.98
Serum LL-37 (ng/ml), median (IQR)	42.0 (28.0-58.5)	46.0 (34.0-60.5)	0.02
Food sensitization†	26 (18.6)	167 (19.1)	0.98

Aeroallergen sensitization†	2 (1.4)	14 (1.6)	0.99
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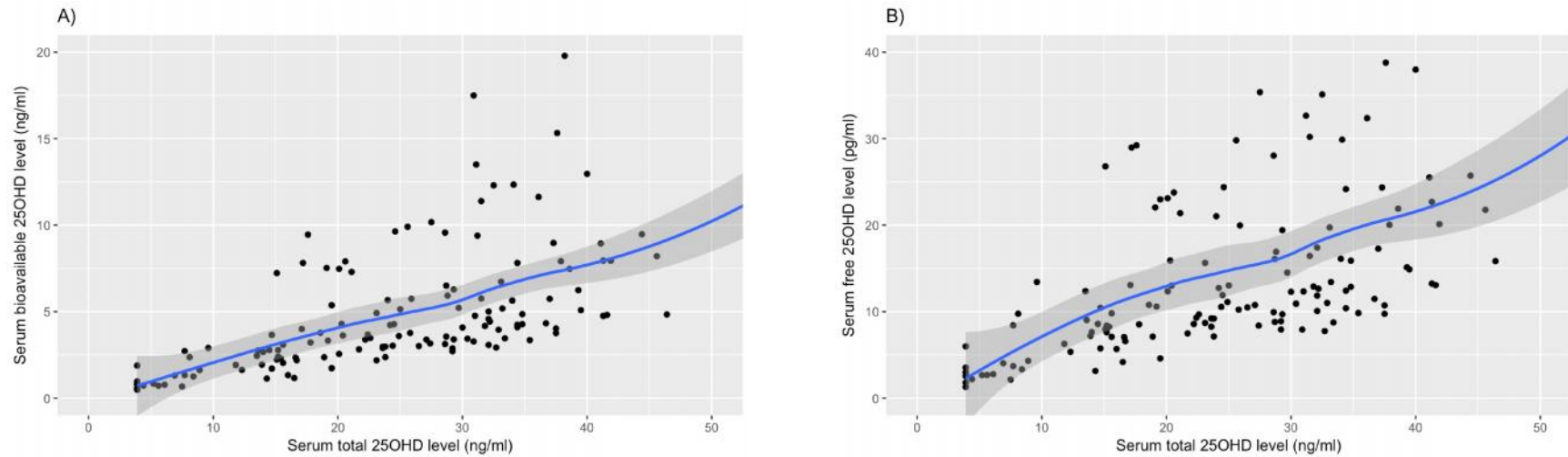
Data are no. (%) of infants unless otherwise indicated. Percentages may not equal 100, because of missingness

Abbreviations: IQR, interquartile range; RSV, respiratory syncytial virus; 25OHD, 25-hydroxyvitamin D

* Defined as a child having cough that wakes him/her at night and/or causes emesis, or when the child has wheezing or shortness of breath without cough

† Defined by having one or more positive values for allergen-specific IgE

Figure S1. Correlations of Serum Total 25-hydroxyvitamin D (25OHD) with Bioavailable and Free 25OHD Levels



A) There was a significant correlation between serum total 25OHD and bioavailable 25OHD levels (Spearman $r=0.73$; $P<0.001$).

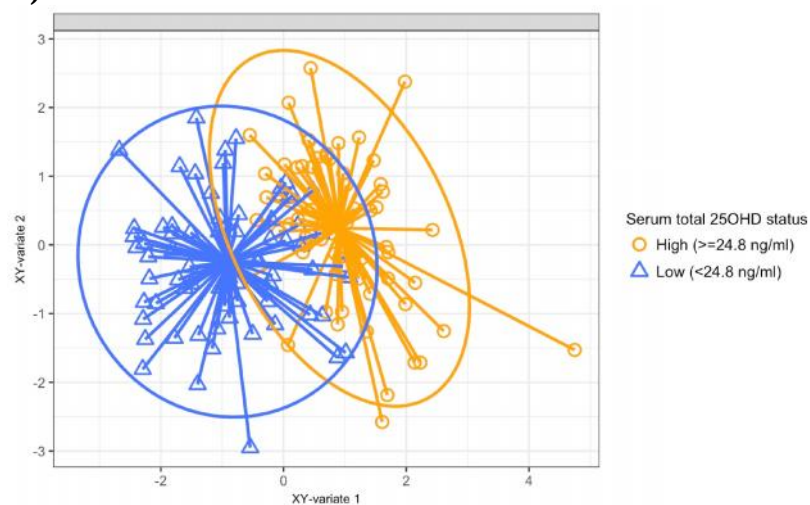
B) There was a significant correlation between serum total 25OHD and free 25OHD levels (Spearman $r=0.66$; $P<0.001$).

The fitted lines represent locally weighted scatterplot smoothed (loess) curve, with 95% confidence intervals.

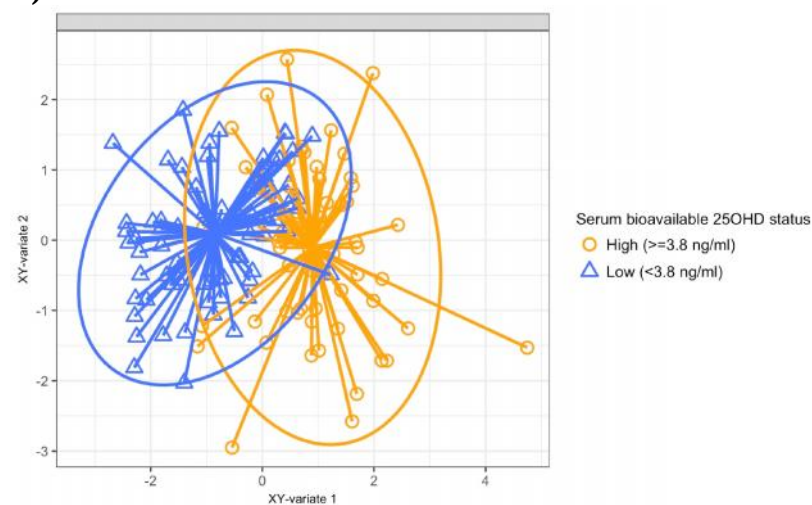
Figure S2. Sparse Partial Least Squares Analysis Score Plots of Serum Metabolome According to 25-hydroxyvitamin D levels

Each dot represents the global serum metabolomic profile of a single patient by plotting the component scores in the smaller subspace spanned by latent variables of sparse partial least squares (sPLS) model. The scatter plot indicates the similarities and dissimilarities in the global metabolomic profile between patients, grouped by serum **A)** total, **B)** bioavailable, and **C)** free 25OHDs (defined by the median values). The eclipses are 95% confidence intervals. The arrows start from the centroid of each group and end for each patient belonging to each group.

A)



B)



C)

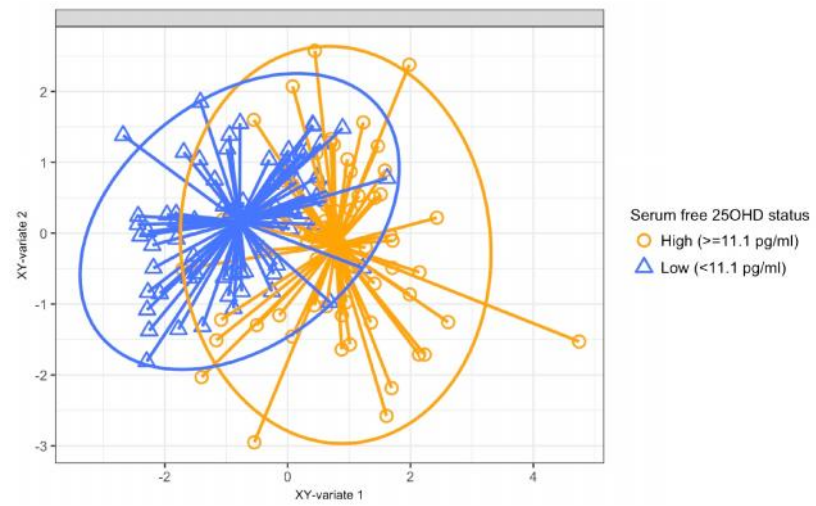


Figure S3. Loading Plot of Serum Metabolites Related to Serum 25-hydroxyvitamin D Levels Among Infants with Bronchiolitis

The plot represents the loading coefficient of 20 serum metabolites that were selected from the sparse partial least squares (sPLS) regression model. The 20 metabolites were ranked according to their contribution to lower serum 25OHD (total, bioavailable, and free) (top to bottom).

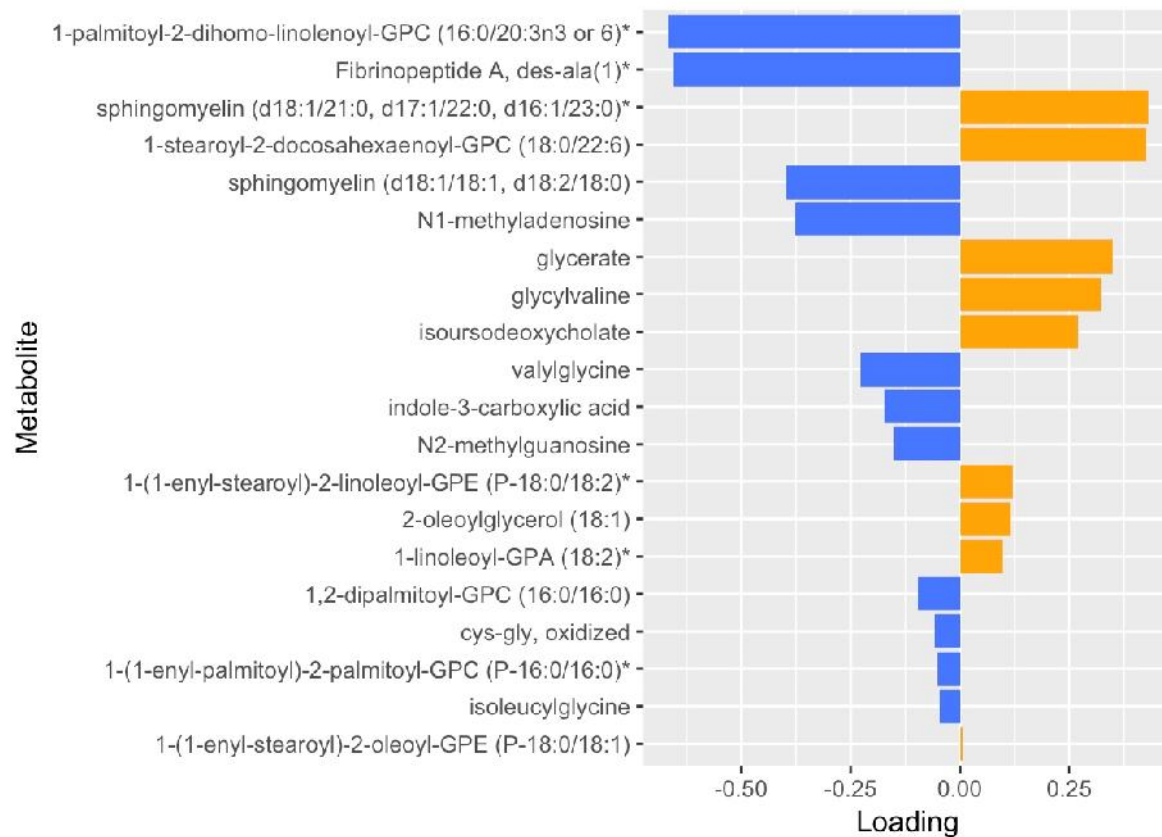


Figure S4. Receiver Operating Characteristic (ROC) Curve of the 20-metabolite Model on the Risk of Positive Pressure Ventilation Use

The ROC curve estimates the discrimination ability of the selected 20 metabolites on the risk of PPV use. The area-under-the estimated receiver operating characteristic curve (AUC) was 0.92, indicating that a high discrimination ability of the 20-metabolite model.

